

Fertilization Success in Marine Invertebrates: The Influence of Gamete Age

MARK ELLIOTT WILLIAMS AND MATTHEW GRAEME BENTLEY*

Gatty Marine Laboratory, The University, St Andrews, Fife, KY16 8LB UK

Abstract. Gamete age has been postulated to be unimportant to the fertilization ecology of marine invertebrates. However, recent research suggests that, for some species at least, it may have a direct impact upon fertilization success. We present comparative data on the influence of gamete age on fertilization and development success in several marine invertebrates: the polychaetes *Arenicola marina* and *Nereis virens* and the asteroid echinoderm *Asterias rubens*. Oocytes are much longer lived in the polychaetes than in the echinoderm, with *A. marina* oocytes still capable of fertilizing and developing normally 96 h post-spawning. Developmental abnormalities and failure to reach blastula tend to occur well before the fertilizable life of the oocytes has expired. Sperm are similarly longer lived in the polychaetes; however, fertilizing capacity is markedly reduced following incubation in conspecific egg-conditioned seawater. These results are discussed in terms of the fertilization strategies of the three species. We further suggest that, for *A. marina* at least, longer-lived sperm and eggs are central to the fertilization strategy of this species.

Introduction

Whether the length of time that sperm and eggs are viable following spawning affects the reproductive success of broadcast spawning species is a subject for debate. The view of workers such as Levitan *et al.* (1991) is that dilution of gametes below fertilizable concentrations occurs well before the viable life of the gametes has expired, leading to the hypothesis that gamete longevity is not important to the fertilization ecology of free-spawning species. Yund (2000)

suggests, however, that adaptations to reduce sperm limitation may lead to high levels of fertilization success, although sperm dilution effects remain undoubtedly important for certain shallow subtidal free-spawning echinoderms and molluscs. Circumstances in which gamete longevity is combined with reduced gamete dilution due to the viscous nature of the fluids in which gametes are released may increase fertilization levels in free-spawning species. This has been demonstrated recently in *Strongylocentrotus droebachiensis* using laboratory investigations (Meidel and Yund, 2001). A different situation exists in the brooding starfish *Leptasterias polaris*, in which sperm deposited on the substratum are not dispersed, but remain quiescent until activated by female spawning (Hamel and Mercier, 1995). In such cases, longer-lived sperm would be a distinct advantage. Sperm longevity is also important in some internally fertilizing ascidians where females filter large volumes of seawater, which can result in the collection and concentration of diluted sperm to effect a high level of fertilization many hours after male spawning (Bishop, 1998).

This study investigates egg and sperm longevity of the polychaetes *Arenicola marina* and *Nereis virens* and the asteroid *Asterias rubens*, which are all species that have seasonal reproduction. *Arenicola marina* and *Asterias rubens* are annual iteroparous species, while *N. virens* is semelparous. The findings will be discussed in an ecological context. The mediation of sperm activity by egg compounds will also be examined to a certain extent for *Arenicola marina* and *Asterias rubens*.

In most populations of the lugworm *Arenicola marina*, spawning is epidemic, cued by environmental and endogenous factors (Bentley and Pacey, 1992; Watson and Bentley, 1998; Watson *et al.*, 2000), and takes place over only a few days in late autumn in most localities (Williams *et al.*, 1997). Male and female lugworms exhibit different spawning strategies. Females spawn their oocytes into the U-

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* To whom correspondence should be addressed. Current address: Department of Marine Sciences and Coastal Management, University of Newcastle, Ridley Building, Claremont Road, Newcastle upon Tyne, NE1 7RU UK. E-mail: m.g.bentley@ncl.ac.uk

shaped burrow, which is irrigated on the flood tide, drawing sperm-enriched water into the fertilization site. Males release sperm puddles onto the surface of the sediment at low water, ejecting small streams of sperm from the tail shaft of the burrow. The sperm coalesce to form a dense puddle with a highly cohesive, oily consistency. In this state the sperm are quiescent (Pacey *et al.*, 1994a). On the flood tide, the sperm puddles are dispersed across the beach, affording females the opportunity to "collect" sperm for fertilization. Field studies of fertilization success indicate that it is highly variable, between 0% and 100% (Williams *et al.*, 1997).

The king ragworm, *Nereis virens*, spawns only once at age 2–5 years, during periods of spring tides in early spring along the East Coast of Scotland. Sperm develop as tetrads, and ripe males are characterized by having free-swimming sperm within the coelomic cavity. Spawning is characterized by swarming, when ripe males leave the sediment and swim actively in the water column releasing gametes (Bass and Brafield, 1972), and the female remains in the substratum depositing her eggs on the surface of the sediment.

Asterias rubens generally spawns during spring in northern European waters, although more northerly populations spawn in the late spring or early summer (Nichols and Barker, 1984). Oocyte maturation and spawning is initiated by 1-methyl adenine (Kanatani, 1979). During spawning, *A. rubens* adopts a spawning posture typical of many asteroids. Waves of contraction pass along the arms of the starfish and the central disc is raised several centimeters off the substratum so that the starfish is supported on the tips of its arms.

Materials and Methods

Collection and maintenance of experimental animals

Mature specimens of *Arenicola marina* were collected by digging from an intertidal sandflat at Red Wharf Bay, Anglesey, North Wales (53°18.6'N 4°12.0'W). This population spawns epidemically during spring tides in early December, and ripe individuals (responsive to endocrine manipulation to stimulate spawning) were available from mid-November. In the laboratory, worms were sexed and housed individually in polyethylene pots with fresh filtered seawater in a controlled temperature room under ambient temperature and illumination. The worms were left for at least 24 h before use in experiments to allow gut contents to be voided, and the water was changed daily.

Gravid individuals of *Nereis virens* were provided by Seabait Ltd. (Ashington, Northumberland) and collected by digging from a designated broodstock bed during late February and March. In the laboratory, the animals were stored in polyethylene pots with a layer of gravel and 0.5-mm diameter holes drilled into the walls and lid. The pots were then placed into single-sex, flow-through tanks and maintained at 5°C with ambient illumination. Using farmed animals rather than field-collected specimens provides ani-

mals at a known stage of reproductive development. Animals at the peak of gamete development (100% fertilization and development to blastula, free sperm in coelomic cavity, and some spontaneous spawning) were selected for use in experiments.

Specimens of *Asterias rubens* were collected from fishing creels set at a number of sites around the East Coast of Fife, Scotland. They were stored in large tanks (1 m × 3 m × 1 m) with a continuous flow-through of fresh seawater and maintained under conditions of ambient temperature and illumination. Starfish were used within a week of collection.

Spawning induction and collection of gametes

Arenicola marina. Mature oocytes were collected from animals induced to spawn by injection of homogenized prostomia from mature females, as described by Pacey and Bentley (1992). Each recipient female was injected with one prostomial equivalent of homogenate and placed into fresh seawater to spawn. Upon spawning, excess water was decanted, and the eggs were washed twice by resuspending in 100 ml sterile filtered seawater (SFSW). They were then allowed to settle out, and a sample was transferred to microcentrifuge tubes. The density of settled eggs in the tubes was determined by counting microliter volumes under a compound microscope.

Sperm maturation through the injection of 8,11,14-eicosatrienoic acid. To induce spawning, each gravid male was injected with the sperm maturation factor 8,11,14-eicosatrienoic acid (Sigma), using an optimal concentration of $13 \mu\text{g} \cdot \text{g}^{-1}$ body mass (for full details see Pacey and Bentley, 1992). A 200- μl Gilson pipette was used to collect sperm dry (*i.e.*, undiluted) as it emerged from the nephromixia. The sperm samples were placed into microcentrifuge tubes and left for a few minutes to allow them to coalesce to maximal concentration; the excess water was then pipetted off.

In vitro maturation of coelomic sperm. Sperm were activated *in vitro* by incubation in appropriate concentrations of 8,11,14-eicosatrienoic acid (Pacey and Bentley, 1992). Samples, about 200 μl in volume, of coelomic fluid were withdrawn from the coelomic cavity of gravid males and placed in microcentrifuge tubes. This allowed multiple sampling from each male. Coelomic samples were incubated with equivalent volumes of $1 \times 10^{-4} M$ 8,11,14-eicosatrienoic acid. They were then shaken by hand and left for 30 min at 8°C to mature. The mature sperm typically coalesced into oily droplets at the bottom of the tube, and the excess fluid was removed. Mature sperm had the same fertilizing capacity as normally spawned sperm. This technique was used when large numbers of small samples of sperm were required and when only the eggs were the subject of investigation.

Nereis virens. Samples of oocytes were withdrawn from

the coelomic cavity with a disposable 1-ml syringe. The oocyte sample was expelled into about 100 ml SFSW and allowed to settle before being washed again in the same volume. Samples of eggs were then transferred to microcentrifuge tubes, and the density of settled eggs was determined. Coelomic sperm were withdrawn using a syringe and stored dry (*i.e.*, undiluted) in microcentrifuge tubes at 8°C until required.

Asterias rubens. Mature oocytes were collected following injection of 1-methyl adenine (1-MeAde) into ripe individuals of *Asterias rubens*. Fresh starfish were collected from the aquarium and placed in about 100 ml SFSW in glass bowls. One to two ml of 10^{-4} M 1-MeAde (prepared in seawater) was injected through the aboral surface of one of the arms. This gave an approximate concentration in the animals of 1×10^{-6} M 1-MeAde. Mature oocytes were collected from the bowl, washed twice in SFSW, and transferred to a beaker with about 100 ml SFSW. Settled egg density was determined as described above.

Asterias rubens sperm were also collected by dissection of gonadal tissue. Pieces of excised testis were placed in a small petri dish containing 10 ml SFSW. Gentle probing with a pipette tip led to the extrusion of sperm from the tissue, which was collected dry using a 200- μ l Gilson pipette. This technique was used only when eggs were under test. Sperm collected following spawning induction were used during investigations of sperm performance.

Oocyte age

Mature oocytes were harvested as described above. Approximately 10^6 eggs from each female were transferred to separate autoclaved beakers containing 1 l of SFSW. Sampling 1 ml of the mixed egg suspensions yielded between 500 and 1000 eggs. The beakers were then stored at 8°C for the duration of the experiment, and the water was carefully changed every 4–8 h with fresh SFSW to maintain oxygen tensions. For *Arenicola marina* and *Asterias rubens*, eggs from five females were used; for *Nereis virens*, eggs from three females were used.

Fertilization assay

To discern any decline in fertilization success, it was necessary to fertilize the eggs with the minimum quantity of sperm required to achieve 100% fertilization. The fertilization assay was conducted by pipetting 1 ml of 5×10^5 sperm \cdot ml $^{-1}$ directly over the eggs in the fertilization chamber. This gave an overall sperm concentration in the 20-ml volume of 2.5×10^4 sperm \cdot ml $^{-1}$, and 100% fertilization was recorded; incidences of abnormal development at time 0 (possibly attributable to polyspermy) were rare. Any fall in fertilization success (and hence gamete fitness) was therefore immediately apparent.

Egg longevity

For *Arenicola marina*, fresh sperm suspension was prepared *in vitro* from at least five males at each time point. The suspension was examined under the microscope to ensure the presence of free-swimming sperm; concentrations were determined using a hemocytometer. Suspensions from each of the males were then pooled to give a final concentration of 10 ml of 5×10^5 sperm \cdot ml $^{-1}$. For males of *Asterias rubens*, small pieces of testis were excised from one arm of each of five males, and sperm were extracted as described above. Each of the five pairs of testes was sampled only once. After collection, the sperm were treated as for *Arenicola marina*. Sperm were collected from *Nereis virens* males directly from the coelomic cavity as described above, and stock solutions of 10 ml of 5×10^5 sperm \cdot ml $^{-1}$ were pooled from at least three males. The fertilizing capacity of the ragworm sperm was tested. Use of these techniques permitted each male to be used several times during the experiment.

Eggs from each species under test were withdrawn from five females as described earlier. Fertilizations were conducted in sterile 25-ml petri dishes. Three replicate fertilizations were conducted for each of the five females at all of the time points used. At each time point, 500–1000 oocytes were pipetted in 1 ml of seawater from the storage beakers into the appropriate petri dish that contained 18 ml SFSW. One milliliter of pooled sperm suspension was then added to the petri dish to give a final volume of 20 ml and a final sperm concentration of approximately 2.5×10^4 sperm \cdot ml $^{-1}$; the petri dishes were then left for 24 h at 8°C. Fertilization success was assessed by examining 150 eggs from each of the dishes. Oocytes were recorded as fertilized with normal development, fertilized with abnormal development, or unfertilized. Abnormal development was defined as embryos failing to develop or degenerating. Allowing such eggs to continue to develop revealed only further degeneration.

Sperm longevity at 10^5 sperm \cdot ml $^{-1}$ and 10^9 sperm \cdot ml $^{-1}$

Gamete handling. Fresh sperm were collected dry from each of five male *Arenicola marina*, five *Asterias rubens*, and three *Nereis virens*; and stock suspensions of 20 ml 5×10^9 sperm \cdot ml $^{-1}$ were prepared in autoclaved glass petri dishes. Aliquots of each of the sperm suspensions were taken and serially diluted to provide 200 ml of sperm suspension at a concentration of 5×10^5 sperm \cdot ml $^{-1}$. These were then stored at 8–10°C for the duration of the experiment. Oocytes were collected from at least three females of each of the species and treated as described above. Fresh oocytes were collected from *Arenicola marina* every 24 h, and from *Asterias rubens* and *Nereis virens* at each fertilization point.

Fertilizations were conducted in sterile 25-ml petri dishes. At each time point, 18 ml of SFSW was pipetted into

the fertilization dishes together with 1 ml containing approximately 500–1000 fresh oocytes. One milliliter of the 5×10^5 sperm \cdot ml $^{-1}$ suspension was pipetted into the appropriate petri dish over the oocytes to give a final concentration of 2.5×10^4 sperm \cdot ml $^{-1}$. One-hundred microliter samples of the 5×10^9 sperm \cdot ml $^{-1}$ suspensions were taken and serially diluted to a concentration of 5×10^5 sperm \cdot ml $^{-1}$, 1 ml of which was pipetted into the appropriate petri dishes. Three replicate fertilizations were performed for each male and treatment used. The fertilization dishes were then left at 8–10°C for at least 18 h. Fertilization success was scored by sampling 150 eggs from each petri dish for evidence of cleavage.

Effect of egg-derived compounds upon sperm longevity. Eggs were collected from *Arenicola marina* and *Asterias rubens* 12 h before the beginning of the experiment from each of five females by injection of appropriate spawning hormones, as described above. The oocytes were gently washed twice in SFSW, pooled, and their density determined. Approximately 3×10^6 eggs were then carefully pipetted into an autoclaved beaker containing 300 ml of SFSW. The suspension was incubated for 12 h at 8°C, and then the upper 200 ml of water was slowly decanted from the eggs and passed through a 60- μ m Nitex mesh. No eggs were observed on the mesh. The water was filtered to 0.2 μ m. The filtrate was used subsequently to observe possible effects of egg-derived compounds on sperm longevity (see below). Eggs from *Asterias rubens* were used for only 3–4 h before being replaced by fresh samples. However, oocytes collected from *Arenicola marina* were used throughout the experiment and were stored as described above. The egg concentration was determined and the volume of water containing 500–1000 eggs calculated.

Sperm were collected dry from each of five males, and fertilization assays were conducted as above. Appropriate aliquots of sperm from each male were then taken and pipetted individually into five crystallizing dishes containing 40 ml of the egg water (filtrate), to give a final concentration of 5×10^5 sperm \cdot ml $^{-1}$. As a control, further aliquots of sperm were taken from each sample and pipetted into crystallizing dishes with 40 ml of 0.2- μ m-filtered seawater that had been left for 12 h at 8°C.

Results

Oocyte age

The percent fertilization and developmental success of oocytes from *Arenicola marina* aged over 5 days are shown in Figure 1A. Fertilization success remained around 100% for up to 72 h but then began to fall rapidly, with only a quarter of oocytes being fertilized at 96 h and almost none at 120 h. Developmental capacity was not diminished to any great extent until 96 hours post-spawning (Fig. 2B). This

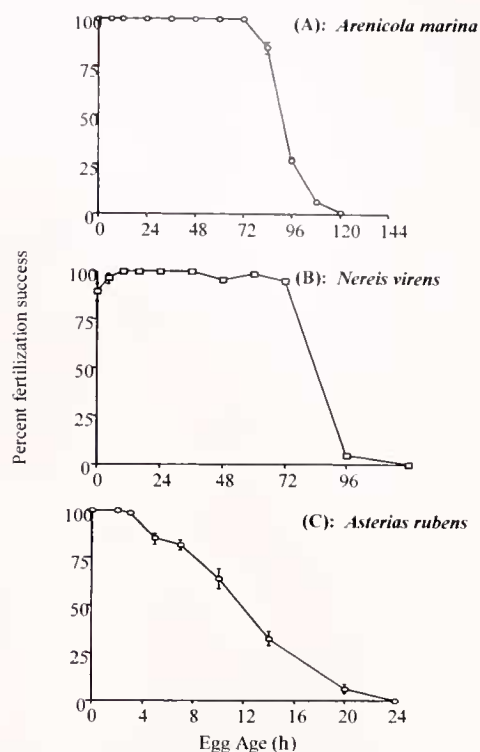


Figure 1. Oocyte fertilization success at a number of time intervals following spawning in *Arenicola marina* (A), *Nereis virens* (B), and *Asterias rubens* (C). Note the difference in timescale over which oocytes were viable for each of the species. Standard errors were calculated from the arcsine-transformed percentage data and back-transformed for presentation.

increase in abnormal development before blastula coincided with the greatest drop in fertilization success.

Most oocytes of *Nereis virens* could be fertilized for at least 72 h after extraction from the coelomic cavity (Fig. 1B); however, the developmental capacity decreased throughout this period so that from 60 h on, only about 10% of the oocytes developed to blastula (Fig. 2B). In contrast to the polychaetes, *Asterias rubens* showed 100% fertilization of oocytes for only the first 4 h after spawning (Fig. 1C). Thereafter, fertilization success fell to zero by 24 h. Cases of abnormal development rose gradually with increasing age (Fig. 2C).

Sperm age

Arenicola marina sperm stored at 10^5 sperm \cdot ml $^{-1}$ retained a high fertilization capacity up to 60 h post-spawning, before dropping to almost zero at 86 h (Fig. 3A). The same graph shows the fertilization success of sperm stored at 10^9 sperm \cdot ml $^{-1}$. Success remained high throughout the experiment and continued beyond the time shown in the figure. When examined, sperm stored at 10^9 sperm \cdot ml $^{-1}$ had

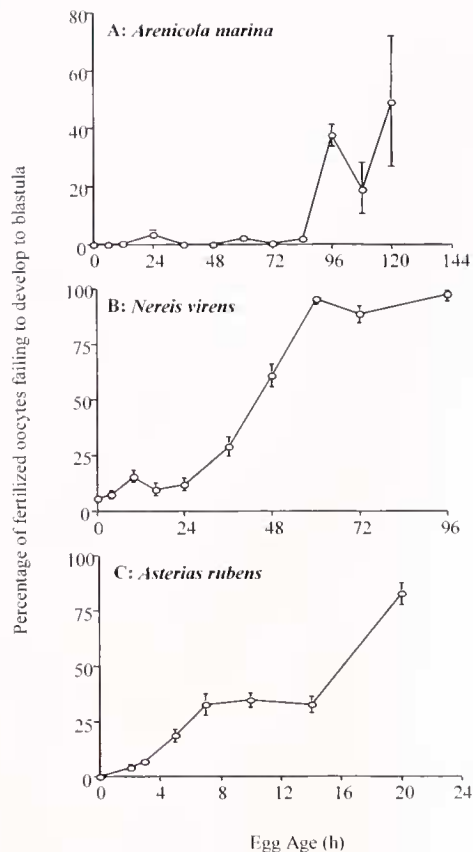


Figure 2. Percentage of oocytes failing to develop to blastula when fertilized at several time intervals following spawning, from *Arenicola marina* (A), *Nereis virens* (B), and *Asterias rubens* (C). Note the difference in timescale over which oocytes were viable for each of the species. Standard errors were calculated from the arcsine-transformed percentage data and back-transformed for presentation.

coalesced into oily droplets at the bottom of the petri dish, and in this state it was quiescent.

Nereis virens sperm stored at the higher concentration was similarly long lived. However, sperm stored at 10^5 sperm \cdot ml $^{-1}$ rapidly lost the ability to fertilize, falling to almost zero after 24 h (Fig. 3B). In contrast, the fertilizing capacity of *Asterias rubens* sperm stored at 10^9 sperm \cdot ml $^{-1}$ and 10^5 sperm \cdot ml $^{-1}$ was found to be similar (Fig. 3C). At each of the time points, success was slightly greater for the sperm stored at the higher concentration, but both successes fell to approximately zero after 26 h.

Sperm stored in egg water

It is apparent that egg water had a marked effect upon the fertilizing capacity of sperm for both *Arenicola marina* (Fig. 4A) and *Asterias rubens* (Fig. 4B). The fertilization capacity of *Arenicola marina* sperm stored in egg water fell to zero after 28 h, whereas that stored in SFSW remained at 100% (data were the same as in Fig. 3A). The fall in

fertilization success of *Asterias rubens* sperm was even more dramatic, dropping to zero after 8 h. The fertilization success of sperm stored in SFSW is comparable to the data presented in Fig. 3C.

Discussion

The longevity of unfertilized oocytes has a marked impact upon fertilization success. This paper demonstrates that there is a finite period following spawning in which the oocyte is capable of being fertilized and subsequently developing to at least the blastula stage. The choice of blastula/early gastrula stage as a cut-off point in the experiments was an arbitrary decision based on the likelihood that artifacts associated with nurturing large batches of larvae might influence the results. Further development may have been

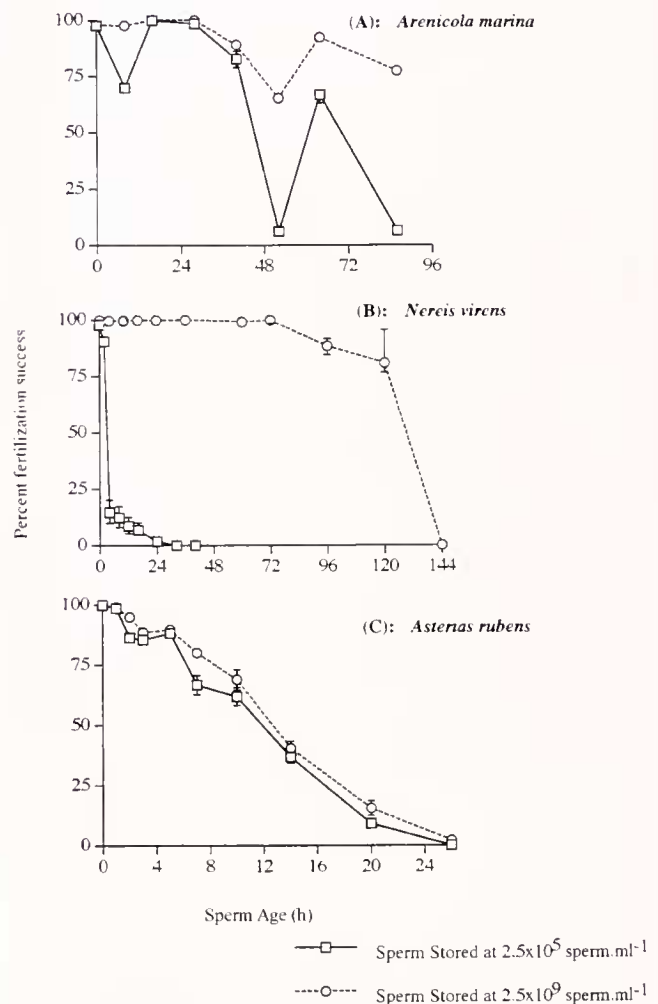


Figure 3. Fertilizing capacity of sperm from *Arenicola marina* (A), *Nereis virens* (B), and *Asterias rubens* (C) stored and aged at two concentrations. Note the difference in timescale over which oocytes were viable for each of the species. Standard errors were calculated from the arcsine-transformed percentage data and back-transformed for presentation.

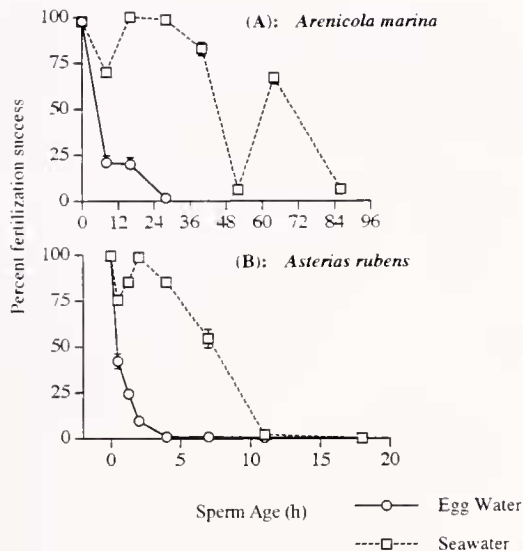


Figure 4. Fertilizing capacity of dilute sperm from *Arenicola marina* (A) and *Asterias rubens* (B) stored either in seawater or in conspecific-egg-conditioned seawater. Note the difference in timescale over which oocytes were viable for each of the species. Standard errors were calculated from the arcsine-transformed percentage data and back-transformed for presentation.

impeded for some embryos as a consequence of oxygen tensions or biotic factors (e.g., infection). The blastula/early gastrula stage, which lasts for a number of hours, also allowed the success (or otherwise) of early embryonic development to be examined and scored without the need for fixation of the samples.

Very few data exist on the longevity of unfertilized gametes among marine invertebrates. That there is a limited life span of gametes was recognized by several early workers (i.e., Lillie, 1915). Recent work has quantified the length of time that gametes are viable, but as Benzie and Dixon (1994) point out, this has not been discussed in an ecological context. It is generally reported that sperm become senescent more rapidly than eggs in free-spawning invertebrates, their longevity being measured from minutes to 1 or 2 hours (see Levitan, 1995; Table 1). This extends across a

range of invertebrate groups including chelicerates (Brown and Knouse, 1973), echinoids (Pennington, 1985; Levitan *et al.*, 1991), asteroids (Benzie and Dixon, 1994), bivalve molluscs (André and Lindegarth, 1995), and hydroids (Yund, 1990).

Oocyte longevity

There are marked differences in the oocyte longevity of the species studied here. *Arenicola marina* oocytes remain fertilizable and capable of developing to blastula for more than 96 h (Figs. 1 and 2). Oocytes from *Nereis virens* remain fertilizable for a similar length of time, but the probability of producing viable offspring decreases to almost zero after 48 h. In contrast, the fertilization capacity of *Asterias rubens* oocytes falls to zero after 24 h, while cases of abnormal development rise steadily throughout. Although there was no evidence for treatment effects on fertilization rates in this study, it is possible that absolute fertilization rates differ from those given. Most of the comprehensive studies of the effect of gamete age upon fertilization success fail to record the length of time that eggs are viable. They simply state that the fall in fertilization success is attributable to a drop in sperm viability and that eggs remain fertilizable for a short time after the sperm has senesced. The oocyte longevity of *A. rubens* is comparable to the qualitative observation by Benzie and Dixon (1994) that oocytes of the starfish *Acanthaster planci* are fertilizable for "a few hours." André and Lindegarth (1995) investigated the independent effect of egg age for the bivalve *Cerastoderma edule*. They reported that eggs could give rise to normally developing embryos for less than 5 h post-spawning.

Arenicola marina oocytes have a remarkable longevity compared to those of the free-spawning species mentioned above and are comparable to the eggs of the ascidian *Ascidia mentula*, where fertilization and development proceeds normally for 96 h after extraction (Havenhand, 1991). Such longevity is not reported elsewhere for externally fertilizing marine invertebrates, and little is known of the factors that determine longevity of oocytes. Internal energy

Table 1

Lengths of time that dilute sperm remain viable at concentrations comparable to those in this paper in some of the species for which sperm longevity has been extensively studied

Species	Viable time (h)	Temp (°C)	Reference
<i>Arenicola marina</i>	70+	10	This study
<i>Nereis virens</i>	24	10	This study
<i>Cerastoderma edule</i> (bivalve mollusc)	5	20	André and Lindegarth, 1995
<i>Asterias rubens</i>	24	10	This study
<i>Acanthaster planci</i> (asteroid echinoderm)	5	28	Benzie and Dixon, 1994
<i>Strongylocentrotus droebachiensis</i> (echinoid echinoderm)	2	14	Pennington, 1985
<i>Strongylocentrotus franciscanus</i> (echinoid echinoderm)	2.5	12	Levitan <i>et al.</i> , 1991

stores will most likely be involved, and the oocytes of both *Arenicola marina* and *Nereis virens* are considerably larger than those of *Asterias rubens*. Following extrusion from the body cavity, the oocytes will be exposed to a range of pathogenic bacteria and toxic compounds. Toomey and Epel (1993) describe embryos of the infaunal echinuran *Urechis caupo* as having a well-developed system for removing toxic compounds that is absent in free-spawned sea urchin embryos. In addition, antibiotic and antifungal resistance is described for the mollusc *Aplysia kurodai* (Iijima *et al.*, 1995). It is likely that the oocytes of *Arenicola marina* will have well-developed defense mechanisms to enable them to remain viable over a longer period of time in the sediment.

Sperm longevity

The results presented here for the polychaetes (Fig. 3A, B) are in accordance with a number of studies on echinoderms (Chia and Bickell, 1983; Levitan *et al.*, 1991; Benzie and Dixon, 1994). The longevity of a sperm suspension is related to sperm concentration; this has been attributed to the rate of consumption of oxygen. The fact that concentrated sperm respire at a lower rate than dilute sperm—termed the respiratory dilution effect (Chia and Bickell, 1983)—has been clearly demonstrated for the echinoid *Strongylocentrotus franciscanus* (Levitan *et al.*, 1991) and the asteroid *Acanthaster planci* (Benzie and Dixon, 1994). *Asterias rubens* sperm was only slightly longer lived at the higher sperm concentration (Fig. 3C). Despite not being assessed over a wide range of concentrations, the dilute (10^5 sperm \cdot ml $^{-1}$) sperm samples for all the species studied here were considerably longer lived than those of some of the species studied elsewhere at comparable concentrations (see Table 1). The sperm longevity of the species presented in this table is less than 5 h, whereas sperm from *Arenicola marina* remains viable for more than 72 h (though at a reduced fertilization success after 48 h). Further, the quiescence of concentrated *A. marina* sperm was shown by Pacey and Bentley (1992).

Following release from the male, sperm from many species require an activation step, which in free-spawning invertebrates is provided by dilution in seawater. Dilution raises the pH of the sperm to that of seawater; this has been shown to activate the spermatozoa of *Arenicola marina* (Pacey *et al.*, 1994a). Dilution also leads to a raised respiration rate and a more active swimming behavior. There is a finite "respiratory life" of a spermatozoon, with those stored at low density having a shorter half-life than more concentrated sperm (Chia and Bickell, 1983). Studies on the swimming behavior of *A. marina* sperm have shown that it has intermittent periods of quiescence, which may be modulated by light radiation (Pacey *et al.*, 1994b). This has also been described for tunicates (Brokaw, 1984) and some sea

urchins (Gibbons, 1980). Bishop (1998) attributes the longevity of ascidian sperm to its intermittent swimming behavior, which results in the conservation of energy stores; such a mechanism could conceivably facilitate the sperm longevity of *A. marina*. Temperature may also influence sperm longevity by changing respiration rate. In the experiments here, sperm was stored at 8°C, which was close to ambient for the time of year but cooler than the temperatures used in the studies presented in Table 1.

Havenhand (1991) showed that sperm from a solitary ascidian (*Ascidia mentula*) can successfully fertilize oocytes for up to 48 h after release and that eggs are similarly longer lived. This longevity was subsequently confirmed for three other ascidian species (Bolton and Havenhand, 1996; Bishop, 1998). The situation in these ascidians is slightly different from that of other free-spawning invertebrates in that the female retains the eggs and fertilization is facilitated by the female collecting the sperm and brooding the eggs. A high fertilization success occurs at sperm densities that are low (around 10 sperm \cdot ml $^{-1}$), which is in contrast to the results of studies by Levitan *et al.* (1991) and Benzie and Dixon (1994) on echinoderms.

One factor that should be considered when comparing species that have slightly different fertilization strategies is that the term "dilute sperm" is a relative one and that it is functional dilution level that is important. For example, some free-spawning echinoderms require hundreds of thousands of sperm per milliliter in the surrounding water to effect a reasonable fertilization success (Levitan *et al.*, 1991). Conversely, some (male only) free-spawning ascidians capture sufficient sperm from the surrounding water to fertilize eggs at concentrations measured in the tens of sperm per milliliter, long after male spawning has ceased (Bishop, 1998). Comparisons of the impact of dilution on longevity between these species would therefore be tenuous, since the fertilization strategy is clearly different. Within the species used in this study, it is interesting that sperm at the lower concentration are longer lived in *Arenicola marina* than in the others, as this species has a fertilization strategy more akin to the ascidian than are the strategies of the other two species.

Impact of egg-conditioned water on sperm activity

For many years before the phenomenon was unequivocally demonstrated, investigators suspected that chemical attraction and subsequent taxis of a sperm towards an egg occurred in animal phyla. It has, however, been known for over a century in plants, and much is known of the nature of the chemical attractants, in the marine brown algae particularly (Maier and Müller, 1986). Chemotaxis in marine invertebrates was first shown by Dan (1950), in a medusoid cnidarian; extensive work by Miller during the 1960s and 1970s demonstrated chemotaxis in a number of other spe-

cies of Cnidaria (Miller, 1970; 1978). It has since been demonstrated in a number of phyla including molluscs (Miller, 1977; Miller *et al.*, 1994), arthropods (*Limulus* sp., Clapper and Brown, 1980), echinoderms (Miller, 1985), and tunicates (Miller, 1975). Whether such chemotaxis exists in annelids is inconclusive at present.

Chemicals derived from the egg can have three types of effect upon the swimming of the sperm. First, they can initiate swimming (*e.g.*, *Limulus* sp., Clapper and Brown, 1980); second, they can increase swimming velocity; and finally, they can cause the sperm to swim in a directed manner towards the egg (see Miller, 1984, for review of sperm swimming behavior). The outcome of these behaviors is simply to increase the probability of sperm-egg interaction. It is believed that the principal effect of a sperm attractant that is released from the spawned egg is to create a larger surface area for the egg, thus making "larger targets" for passing sperm (see Cosson, 1990). However, the distance over which the attractants are effective will be limited by the constraints of sperm swimming capacity and by hydrodynamic effects.

Incubation of *Arenicola marina* sperm and *Asterias rubens* sperm in egg-conditioned water dramatically reduced their respective half lives. The fertilizing capacity of *Arenicola marina* sperm dropped to zero after little more than a day, and *Asterias rubens* sperm produced almost no fertilization after 4 h. These results are similar to data obtained by Bolton and Havenhand (1996) for the solitary ascidians *Ciona intestinalis* and *Asciidiella aspersa*, whose sperm is usually active for more than 12 h. It seems likely that the egg-derived compounds studied here act by raising the activity and respiration levels of the sperm, in a mechanism similar to that described by Suzuki and Garbers (1984). Bolton and Havenhand (1996) noted that ascidian sperm incubated in egg water swam more often than those incubated in seawater. We found some preliminary evidence to support the hypothesis that egg compounds increase the rate of sperm respiration in *Arenicola marina*, but limitations of the experimental equipment prevented a more rigorous investigation of this hypothesis.

Ecological considerations

Oocyte longevity is probably related to the mode of reproduction of a species. Data gathered from the field indicate that the oocytes of *Arenicola marina* may be spawned early in the spawning period (Williams *et al.*, 1997). Fertilization success is limited by sperm puddle density, and not all oocytes are fertilized after one exposure to sperm. Consequently, longer-lived oocytes confer an advantage by being capable of fertilizing and developing up to 5 days after spawning. We propose that the fertilization strategy of *A. marina* is that the female releases her eggs at the beginning of the spawning period, and that overall

fertilization success is the result of an accumulation of fertilizations over the 4- to 5-day spawning period (see Williams *et al.*, 1997). In comparison, the eggs and sperm of *Nereis virens* and *Asterias rubens* are more likely to be dispersed by water currents before their longevity has expired, according to Levitan (1995). However, the dilution of gametes beyond fertilizable concentrations in the field has been examined for only a few species of echinoderm, and the theory that dilution is more important than longevity may be found not to hold true for a wider range of free-spawning species. Thomas has demonstrated that the viscosity of egg and sperm fluids in several free-spawning echinoderms (Thomas, 1994a) and polychaetes (Thomas, 1994b) is such that dilution is delayed and gamete longevity may still be important.

The longevity of *Arenicola marina* sperm at low densities may also be an adaptation to the fertilization strategy of this species. Sperm are released onto the surface of the sediment at low water, where they coalesce into dense puddles. It may be several hours before they are dispersed by the tide, so the lack of activity in high concentration, which is further enhanced by light (Pacey *et al.*, 1994b), prevents the spermatozoa from expending too much energy while they are remote from the eggs. After the sperm are diluted and carried into the female burrow, there may be a further delay before they encounter an egg, because the rate of irrigation of the female burrow is low. Consequently, interaction between sperm and egg in *Arenicola marina* may occur many hours after either has been spawned, in contrast to most other free-spawning invertebrates.

Egg-derived compounds could play an important role in the fertilization success—and thus the fertilization strategy—of *Arenicola marina*. Stimulatory compounds from the eggs would ensure that sperm were most active when close to an oocyte. Thus, there is a twofold process. The hydrodynamic factors bring the sperm and eggs into brief contact, and the chemicals emanating from the egg stimulate the sperm to respond. The lack of turbulent mixing in the female burrow of *Arenicola marina* and the comparatively long viable life of the sperm may mean that compounds released by the egg that enhance sperm swimming are of particular importance in increasing the probability of encounters between sperm and egg.

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Literature Cited

- André, C. and M. Lindegarth. 1995. Fertilization efficiency and gamete viability of a sessile, free-spawning bivalve, *Cerastoderma edule*. *Ophelia* 43: 215–227.
- Bass, N. R., and A. E. Brafield. 1972. The life cycle of the polychaete *Nereis virens*. *J. Mar. Biol. Assoc. UK* 52: 701–726.
- Bentley, M. G., and A. A. Pacey. 1992. Physiological and environmental control of reproduction in polychaetes. Pp. 443–481 in *Oceanography and Marine Biology: An Annual Review*, Vol. 30. M. Barnes, A. D. Ansell, and R. N. Gibson, eds. UCL Press, London.
- Benzie, J. A. H., and P. Dixon. 1994. The effects of sperm concentration, sperm:egg ratio, and gamete age on fertilization success in crown-of-thorns starfish (*Acanthaster planci*) in the laboratory. *Biol. Bull.* 186: 139–152.
- Bishop, J. D. D. 1998. Fertilization in the sea: are the hazards of broadcast spawning avoided when free-spawned sperm fertilize retained eggs? *Proc. R. Soc. Lond. B* 265: 725–731.
- Bolton T. F., and J. N. Havenhand. 1996. Chemical mediation of sperm activity and longevity in the solitary ascidians *Ciona intestinalis* and *Ascidella aspersa*. *Biol. Bull.* 190: 329–335.
- Brokaw, C. J. 1984. Cyclic AMP-dependent activation of sea urchin and tunicate sperm motility. *Ann. N. Y. Acad. Sci.* 438: 132–141.
- Brown, G. G., and J. R. Knouse. 1973. Effects of sperm concentration, sperm aging, and other variables on fertilization in the horseshoe crab, *Limulus polyphemus* L. *Biol. Bull.* 144: 462–470.
- Chia, F. S., and L. R. Bickell. 1983. Echinodermata. Pp. 545–619 in *Reproductive Biology of Marine Invertebrates Vol. II: Spermatogenesis and Sperm Function*, K. G. and R. G. Adiyodi, eds. Wiley and Sons, New York.
- Clapper, D. L., and G. G. Brown. 1980. Sperm motility in the horseshoe crab, *Limulus polyphemus*. I. Sperm behavior near eggs and motility initiation by egg extracts. *Dev. Biol.* 76: 341–349.
- Cosson, M. P. 1990. Sperm chemotaxis. Pp. 103–135 in *Controls of Sperm Motility: Biological and Clinical Aspects*, C. Gagnon, ed. CRC Press, Boca Raton, FL.
- Dan, J. C. 1950. Fertilization in the medusan, *Spirocodon saltatrix*. *Biol. Bull.* 99: 412–415.
- Gibbons, B. H. 1980. Intermittent swimming in live sea urchin sperm. *J. Cell. Biol.* 84: 1–12.
- Hamel, J.-F., and A. Mercier. 1995. Prespawning behavior, spawning, and development of the brooding starfish *Leptasterias polaris*. *Biol. Bull.* 188: 32–45.
- Havenhand, J. N. 1991. Fertilization and the potential for dispersal of gametes and larvae in the solitary ascidian *Ascidia mentula* Müller. *Ophelia* 33: 1–15.
- Iijima, R., J. Kisugi, and M. Yamazaki. 1995. Antifungal activity of aplysinin-e, a cytotoxic protein of sea-hare (*Aplysia kurodai*) eggs. *Dev. Comp. Immunol.* 19: 13–19.
- Kanatani, H. 1979. Hormones in echinoderms. Pp. 273–307 in *Hormones and Evolution*, Vol. 1, E. J. W. Barrington, ed. Academic Press, London.
- Levitán, D. R. 1995. The ecology of fertilization in free-spawning invertebrates. Pp. 123–156 in *Ecology of Marine Invertebrate Larvae*, L. McEdward, ed. CRC Press, Boca Raton, FL.
- Levitán, D. R., M. A. Sewell, and F.-S. Chia. 1991. Kinetics of fertilization in the sea urchin *Strongylocentrotus franciscanus*: interaction of gamete dilution, age, and contact time. *Biol. Bull.* 181: 371–378.
- Lillie, F. R. 1915. Studies on fertilization. VII: Analysis of variations in the fertilizing power of sperm suspensions of *Arbacia*. *Biol. Bull.* 28: 229–251.
- Mäler, L., and D. G. Müller. 1986. Sexual pheromones in algae. *Biol. Bull.* 170: 145–175.
- Meidel, S. K., and P. O. Yund. 2001. Egg longevity and time-integrated fertilization in a temperate sea urchin (*Strongylocentrotus droebachiensis*). *Biol. Bull.* 201: 84–94.
- Miller, R. L. 1970. Sperm migration prior to fertilization in the hydroid *Gonathyprea loveni*. *J. Exp. Zool.* 175: 493–504.
- Miller, R. L. 1975. Chemotaxis in the spermatozoa of *Ciona intestinalis*. *Nature* 254: 244–245.
- Miller, R. L. 1977. Chemotactic behaviour of the sperm of chitons (Mollusca: Polyplacophora). *J. Exp. Zool.* 202: 203.
- Miller, R. L. 1978. Site-specific sperm agglutination and the timed release of a sperm chemoattractant by the egg of the leptomedusan, *Orthopyxis caliculata*. *J. Exp. Zool.* 205: 385.
- Miller, R. L. 1984. Sperm chemo-orientation in the metazoa. Pp. 275–357 in *Biology of Fertilization*, C. B. Metz and A. Monroy, eds. Academic Press, New York.
- Miller, R. L. 1985. Demonstration of sperm chemotaxis in the Echinodermata: Asteroidea, Holothuroidea and Ophiuroidea. *J. Exp. Zool.* 234: 383–414.
- Miller, R. L., J. J. Mosares, and J. L. Ram. 1994. Species-specific sperm attraction in the zebra mussel, *Dreissena polymorpha* and the quagga mussel, *Dreissena bugensis*. *Can. J. Zool.* 72: 1764–1770.
- Nichols, D., and M. F. Barker. 1984. A comparative study of reproductive and nutritional periodicities in two populations of *Asterias rubens* (Echinodermata: Asteroidea) from the English Channel. *J. Mar. Biol. Assoc. UK* 64: 471–484.
- Pacey, A. A., and M. G. Bentley. 1992. The fatty acid 8, 11, 14 eicosatrienoic acid induces spawning in the male lugworm *Arenicola marina*. *J. Exp. Biol.* 173: 165–179.
- Pacey, A. A., J. C. Cosson, and M. G. Bentley. 1994a. The acquisition of forward motility in the spermatozoa of the polychaete *Arenicola marina*. *J. Exp. Biol.* 195: 259–280.
- Pacey, A. A., J. C. Cosson, and M. G. Bentley. 1994b. Intermittent swimming in the spermatozoa of the lugworm *Arenicola marina* (L.) (Annelida, Polychaeta). *Cell Motil. Cytoskel. et.* 29: 186–194.
- Pennington, J. T. 1985. The ecology of fertilization of echinoid eggs: the consequences of sperm dilution, adult aggregation, and synchronous spawning. *Biol. Bull.* 169: 417–430.
- Suzuki, N., and D. L. Garbers. 1984. Stimulation of sperm respiration rates by speract and resact at alkaline extracellular pH. *Biol. Reprod.* 30: 1167–1174.
- Thomas, F. I. M. 1994a. Physical properties of gametes in three sea urchin species. *J. Exp. Biol.* 194: 263–284.
- Thomas, F. I. M. 1994b. Transport and mixing of gametes in three free-spawning polychaete annelids, *Phragmatopoma californica* (Fewkes), *Sabellaria cementarium* (Moore) and *Schizobranchia insignis* (Bush). *J. Exp. Mar. Biol. Ecol.* 179: 11–27.
- Toomey, B. H., and D. Epel. 1993. Multixenobiotic resistance in *Urechis caupo* embryos: protection from environmental toxins. *Biol. Bull.* 185: 355–364.
- Watson, G. J., and M. G. Bentley. 1998. Action of CMF (Coelomic Maturation Factor) on oocytes of the polychaete *Arenicola marina* (L.). *J. Exp. Zool.* 281: 65–71.
- Watson, G. J., M. E., Williams, and M. G. Bentley. 2000. Can synchronous spawning be predicted from environmental parameters? A case study of the lugworm *Arenicola marina*. *Mar. Biol.* 136: 1003–1017.
- Williams, M. E., M. G. Bentley, and J. D. Hardege. 1997. Assessment of field fertilization success in the infaunal polychaete *Arenicola marina* (L.). *Invertebr. Reprod. Dev.* 31: 189–197.
- Yund, P. O. 1990. An *in situ* measurement of sperm dispersal in a colonial marine hydroid. *J. Exp. Zool.* 253: 102–106.
- Yund, P. O. 2000. How severe is sperm limitation in natural populations of marine free-spawners? *Trends Ecol. Evol.* 15: 10–13.